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A new biocatalytic route to enantiopure *N*-carbamoyl amino acids by fast enzyme screening

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Abstract—The enantioselective enzymatic deamidation of (*rac*)-*N*-carbamoyl amino acid amides (Cbm-AA-NH_2) to enantiopure (*L*)-*N*-carbamoyl amino acids (Cbm-AA-OH) is described for the first time. Via fast screening methods of biocatalysts several proteases like Chirazyme P1, Chirazyme P2 and Subtilisin were identified, which give conversions of up to 47% and >98% ee. This conversion is most productive on aliphatic and primary amino acids. © 2003 Elsevier Science Ltd. All rights reserved.

N-Carbamoyl amino acids are the key-intermediates in the enzymatic hydantoinase–carbamoylase process for the production of amino acids from hydantoins. A well established example is the enzymatic production of *D*-hydroxyphenylglycine as an intermediate of β-lactam antibiotic amoxycillin.[1] Also for aliphatic amino acids like *L*-methionine a process via this hydantoinase–carbamoylase route was developed.[2] Substituted hydantoins are the starting material for this process, which are typically produced by the Bucherer–Bergs reaction from the corresponding aldehyde (Fig. 1).

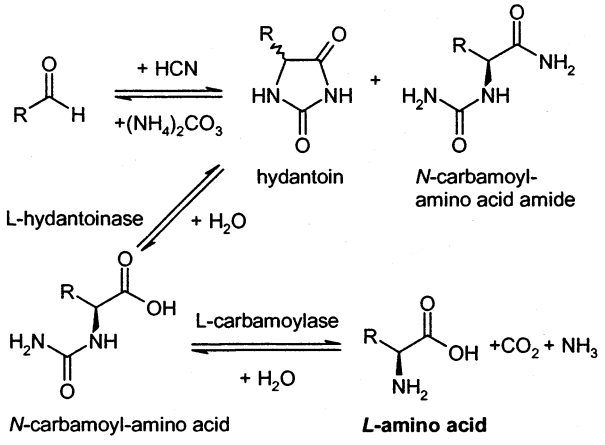

Figure 1.

N-Carbamoyl amino acid amides are an undesired by-product of this reaction. It would be advantageous to have an enzyme that could convert this amide to the desired product by way of selective deamidation. This would increase the overall yield and simplify a process by avoiding the need of purification to remove the amide by-product. An enzymatic process with the advantage of its mild conditions would fit very well with the concept of the whole biotransformation process. Such an enzyme in combination with the carbamoylase would allow for a direct conversion to the free amino acid avoiding isolation steps or further chemical treatment. Furthermore, the enzymatic deamidation of derivatives of amino acid amides is of general interest for the synthesis of several amino acids and protecting group strategy of peptides. It is known that some enzymes from mammalian kidney or pancreas and various microorganisms hydrolyze amino acid amides to the free amino acids.[3] Amidases and proteases like subtilisin hydrolyze *N*-urethyl-protected amino acids and their derivatives.[4] The stereoselective deamidation of *N*-protected (Cbz, Boc, Ac) amino acids was achieved by papain.[5]

However, as far as we know, the enzymatic deamidation of *N*-carbamoyl-protected amino acid amides has never been described in the literature. A further precondition for such an enzymatic reaction in application the hydantoinase/carbamoylase process that it is selective towards the amide group while leaving the *N*-car-

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bamoyl group untouched. Furthermore, an undesired hydantoinase activity must be avoided, as the amidase will be present in a one-pot synthesis together with the hydantoin.

It seemed convenient to screen first those enzymes which are commercially available or otherwise easily available for us. We have therefore built up a library of such enzymes in a 'ready-to-use format', which allows us to validate their activity in a very short time. In order to minimize the preparation time for the screening, this library was installed in a robot-suitable format. Therefore, lyophilized enzymes are stored in crimp seal vials, which are easily handled by a BIOMEK2000 from Beckman–Coulter. Reaction preparations, such as the handling of the substrate and the enzymes as well as the adaptation of the reaction conditions like pH and additives, are robot-assisted. The reaction itself takes place in crimp seal vials, thus minimizing the consumption of enzymes or substrates. Subsequently, the samples are analyzed by HPLC. For the deamidation of *N*-carbamoyl amino acid amides we have chosen a diverse sublibrary of about 40 enzymes to screen, consisting of proteases, acylases, and lipases as these enzyme classes have proven their synthetic usefulness in amino acid chemistry. At first we used *N*-carbamoyl methionine amide (Cbm-Met-NH_2) as substrate for the enzyme screening. For the screening, a solution of *N*-carbamoyl methionine amide (50 μM) and the corresponding enzyme (10 mg/ml) was stirred for 24 h at room temperature (Fig. 2).[6]

Surprisingly, several enzymes with amidase activity towards *N*-carbamoyl methionine amide were found. They are listed in Table 1. The best results were achieved with proteases. The highest conversion of 47% in 24 h is observed with Chirazyme P2 which is equivalent to Novozyme 539, an *endo*-proteinase from *Bacillus*. Further hits are Chirazyme P1, Subtilisins from *Bacillus subtilis* and *Bacillus licheniformis* and to a lower extent even Chymotrypsin. Papain and Trypsine, as well as Newlase and Pepsine were also tested but were not active in this test reaction. The influence of the *N*-protecting group on the enzyme activity can be seen, as Papain, which works very well with urethyl-protected amino acid amides, is not active on carbamoyl-protected amides.[7] Even the lipase from *Candida antarctica* is active in this amidase reaction. Other lipases had low to no amidase activity. Acylases, e.g. from porcine kidney, show only a very low activity. Since we were not able to detect free methionine, all

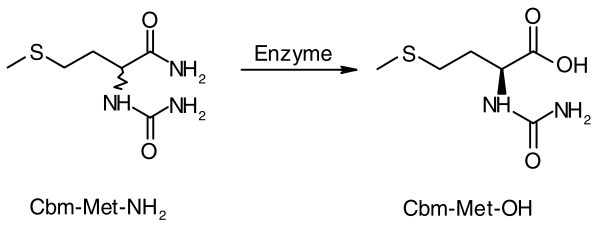

Figure 2.

Table 1. Screening for selective deamidation of *N*-carbamoyl methionine amide

Enzyme	Vendor	Yield (ee) [%]
Proteases		
Chirazyme P1	Roche	21 (>98)
Chirazyme P1 lyo.	Roche	27 (>98)
Chirazyme P2	Roche	47 (>98)
Novozyme 539	Novozymes	47 (>98)
Subtilisin *Bacillus subtilis*	Roche	11 (>98)
Subtilisin *Bacillus licheniformis*	Roche	36 (>98)
Subtilisin *Bacillus licheniformis*	Fluka	23 (>98)
Chymotrypsin A4 bovine pancreas	Roche	12 (>98)
Acylases		
Acylase I porcine kidney	Sigma	4 (n.d.)
Lipases		
Lipase hog pancreas	Fluka	4 (n.d.)
Lipase *Candida antarctica*	Fluka	22 (>98)

n.d.: not determined.

these enzymes were chemoselective to the amide group without cleaving the *N*-carbamoyl group. Next, we investigated the enantioselectivity in this reaction and it was found that all active enzymes showed an ee >98%, where the (*S*)-enantiomer is preferred.

Further experiments were carried out to evaluate the scope and limitations of this racemic resolution. First, the results of the screening were confirmed on a preparative 50 ml scale for Chirazyme P1 with a yield of 33% and ee >98% within 2 days at room temperature. The minimal enzyme:substrate ratio for Chirazyme P2 was determined to 1 mg:4 mg at an enzyme concentration of 2.5 mg/ml. Thereby the conversion for this ratio was lowered from 47 to 23% without affecting the ee. At lower ratios no conversion is observed.

For the integration of amidase active enzymes in the hydantoinase/carbamoylase process for the production of free amino acids it is necessary to check their (unwanted) hydantoinase activity as it is known that proteolytic enzymes are sometimes also hydatoinase active.[8] This was done by comparing the relative amount of carbamoyl methionine formed when a mixture of hydantoin and *N*-carbamoyl methionine amide was used as substrate versus pure *N*-carbamoyl methionine amide.[9] It was found that Chirazyme P1 lyo., *Bacillus licheniformis* Subtilisin from Sigma, Acylase I and *Candida antarctica* lipase show a significant hydantoinase activity and are therefore not suitable for an one-pot process together with the hydantoinase and carbamoylase. On the other hand Chirazyme P2, Novozyme 539, Chirazyme P1 and *Bacillus licheniformis* Subtilisin from Roche have nearly no hydantoinase activity.

Besides methionine also other amino acids react very well as substrates with respect to yield and selectivity as seen in Table 2.[10] Especially derivatives of primary amino acids with aromatic and aliphatic substituents show good results. Chirazyme P1 shows an even better

Table 2. Selective hydrolysis of different *N*-carbamoyl amino acid amides by Chirazyme P1 and Chirazyme P2 at pH 7.5, 22°C and 24 h

Amino acid	Yield (ee) [%]	
	Chirazyme P1	Chirazyme P2
Phenylalanine	47 (>98)	47 (>98)
Tryptophane	44 (>98)	40 (>98)
Leucine	45 (n.d.)	25 (n.d.)
Proline	<1	<1
Phenylglycine	<1	<1

n.d.: not determined.

activity than Chirazyme P2 for aromatic amino acids and leucine. However, this reaction seems to be restricted to primary amino acids with aliphatic substituents, as proline as a secondary amino acid and phenylgylcine with its adjacent phenyl ring fail.

In conclusion we have shown that *N*-carbamoyl amino acid amides are enantioselectively hydrolyzed to *N*-carbamoyl amino acids by a number of proteases and even a lipase. This racemic resolution helps to improve the hydantoinase/carbamoylase process and may also be applied in protecting group techniques of peptide chemistry.

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6. *Experimental conditions*: All commercially available enzymes were purchased or obtained from Roche, Amano, Fluka or Novozymes. HPLC analysis was performed on a HP 1100. Column: Waters terra tm, eluent: H_2O/Acetonitrile/TFA = 985/15/1, flow: 0.1 ml/min, 220 nm, 25°C. Sample preparation: 900 μl H_2O+100 μl reaction mixture. Chiral HPLC was performed on a HP 1100. Column Chirobiotic Ttm Astec, Eluent: Ethanol/H_2O/TFA = 900/100/0.1; 0.4 ml/min, 60 bar, 220 nm.
Enzyme screening: In a crimp seal vial with magnetic stirring bar, *N*-carbamoyl-(*DL*) methionine amide (10 mg ml^{-1}) or the corresponding *N*-carbamoyl amino acid amide was dissolved in 0.5 ml phosphate buffer (50 mM, pH 7.5) and adjusted to pH 7.5 by 1N NaOH. After addition of 5 mg of enzyme the reaction mixture was stirred at 22°C for 24 h and subsequently analyzed by HPLC.
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9. *Test of hydantoinase activity*: In a crimp seal vial with magnetic stirring bar, 0.5 ml of a 17:83 mixture of *N*-carbamoyl-(*DL*) methionine amide and methionine hydantoin (10 mg ml^{-1}) was dissolved in phosphate buffer (50 mM, pH 7.5) and adjusted to pH 7.5 by 1N NaOH. After addition of 5 mg of enzyme the reaction mixture was stirred at 22°C for 24 h and subsequently analyzed by HPLC.
10. *N*-Carbamoyl amino acid amides were prepared according to: Taillades, J.; Rousset, A.; Lasperas, M.; Commeyras, A. *Bull. Soc. Chim. Fr.* **1986**, *4*, 650–658.